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PRINCIPAL INVESTIGATOR: Thipparthi R Reddy, Ph.D.

CONTRACTING ORGANIZATION: Wayne State University-SOM  
Detroit, MI 48201

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14. ABSTRACT Sam68 is a target for <i>Src</i> kinase. It is involved in HIV-1 RNA export. It has been well documented that patients have either lost or have mutations in BRCA1 or BRCA2 tumor suppressor genes are at high risk of developing breast cancers. Thus, the major goal of this application was to investigate whether Sam68 is involved in the export of BRCA1 mRNA. The other goal was to map the BRCA1 mRNA export pathway. To test this objective, we knocked down Sam68 in MCF7 cells. Sam68 knockdown cells expressed reduced levels of BRCA1 mRNA. However, it is not known whether the effect on BRCA1 is due to the transcriptional repression or RNA stability. More evidence came from the Sam68 dominant negative mutant approach, in which BRCA1 protein is repressed. This may account for the RNA destability. Using the Leptomycin B, we have shown that BRCA1 mRNA uses CRM1-dependent pathway. Taken together, our results suggest that Sam68 regulates BRCA1 RNA metabolism.					
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## INTRODUCTION

Breast cancer is the most common and aggressive cancer among the American women aged 50 and above. It is well established that patients either lost or having mutations in BRCA1 or BRCA2 tumor suppressor genes are at high risk of developing breast cancers (Futreal et al. 1994; Miki et al. 1994). BRCA1 was shown to encode a tumor suppressor, a protein that acts as a negative regulator of tumor growth (Thompson and Easton, 2004). However, it is not known whether reduced BRCA1 expression is due to the failure of export of mutant BRCA1 mRNAs to the cytoplasm. In this application, our objective is to determine whether Sam68, an RNA binding protein, is involved in the regulation of BRCA1 at the post-transcriptional level. Sam68 is a target of *c-Src* in mitosis (Taylor and Shalloway 1994; Fumagalli et al. 1994). Although its cellular target is unclear, it was implicated in the nuclear export of human immunodeficiency virus-1 (HIV-1) RNAs (Reddy et al. 1999). Besides its role in HIV RNA export, Sam68 was shown to serve as a substrate for BRK/Sik kinases (breast tumor kinase) (Derry et al. 2001). There are data demonstrating that Breast tumor cell lines express high levels of BRK kinase (Derry et al. 2001). BRK/Sik kinase phosphorylates Sam68 and negatively regulates Sam68-mediated RNA export (Derry et al. 2001). Additionally, Sam68 was shown to interact with CREB-binding protein (CBP) (Hong et al. 2002). CBP acetylates Sam68 and enhances its RNA binding affinity (Babic et al. 2004). CBP regulates the transcriptional activation of ERE-mediated gene expression (Smith et al. 1996). Given that tumorigenic human mammary cancer cells express high levels of acetylated Sam68 (Babic et al. 2004), we reason that Sam68 may regulate the expression of genes, such as BRCA1, involved in tumor cell proliferation at the molecular level (export/stability of BRCA1 RNA). The mutated BRCA1 mRNAs may be unrecognizable by Sam68, and therefore may fail to be exported to the cytoplasm. As a result, the mutant BRCA1 mRNA accumulated in the nucleus may be subjected to degradation, which results either in poor or no expression of BRCA1 protein. The goals of this application are to investigate the extent to which Sam68 is involved in the post-transcriptional regulation of BRCA1 mRNA (i.e., export/stability) and to determine the RNA export pathway of BRCA1 mRNA.

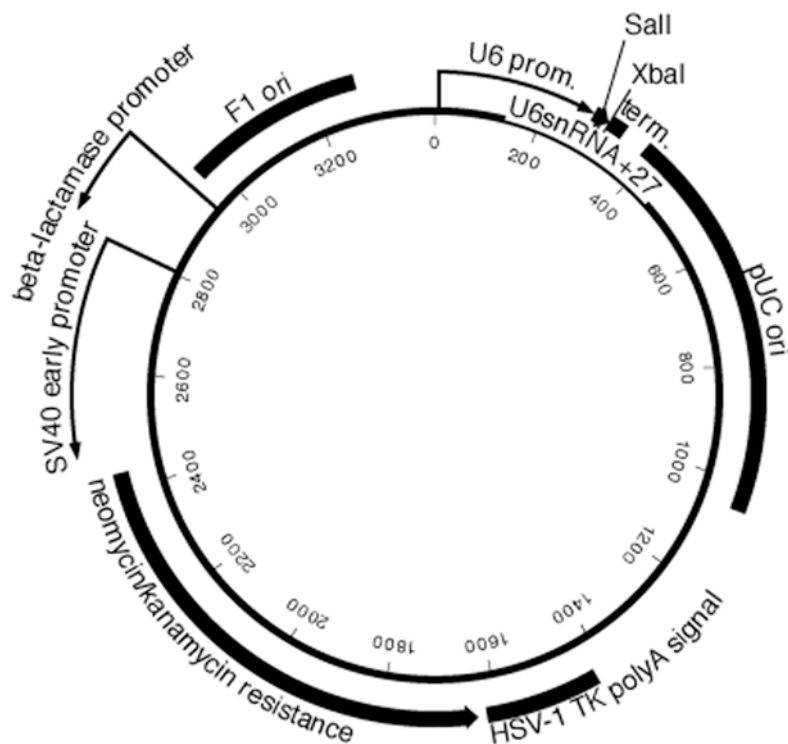
## BODY

***Aim I. Elucidate the role of Sam68 in BRCA1 mRNA export:*** We examined the effect of Sam68 knockdown on BRCA1 expression in MCF7 cells. Employing RNAi strategy, we depleted the expression of Sam68, and examined the reduced levels of Sam68 on BRCA1 mRNA (i. e. expression and export).

### Construction of Sam68-RNAi vector:

We used SuppressorNeo-IMG-800 (Imgenex Corp., San Diego, CA) expression vector to generate the stable Sam68 and IL10 knockdown MCF7 cells. Following primers were annealed and cloned into the *Sa*I and *Xba*I restriction enzyme sites of pIMG-800 to generate pSam68-RNAi and pIL10-RNAi using standard protocol.

Sam68 (forward primer: 5'-TCGAGGGATGATGAGGAGAATTACGAGTACTGGTAATTCTCCTCATC ATCCTTTTT-3' and reverse primer: 5'-CTAGAAAAAGGATGATG-AGGAGAATTACCAGTACTCGTA ATTCTCCTCATCATCCC-3'). IL10 (forward primer: 5'-TCGAGCATACTGCTAACCGACTCCGAGTA CTGGGAGTCGGTTAGCAGTATGTTTTT-3' and reverse primer: 5'-CTAGAAAAACATACTGCAACC GACTCCCAGTAC TCGGAGTCGGTTAGCAGTATGC-3').



SAM68-RNAi

5' -GGATGATGAGGAGAATTAC-3'  
3' -CCTACTACTCCTCTTAATG-5'

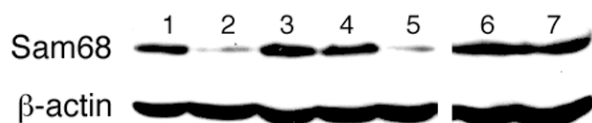
IL10-RNAi

5' -CATACTGCTAACCGACTCC-3'  
3' -GTATGACGATTGGCTGAGG-5'

**Figure 1: Schematic diagram of RNAi expression vector (IMG-800), and Sam68-RNAi and IL10-RNAi target sequences.**

#### Creation of stable Sam68 knockdown MCF7 cells:

The main objective of this study was to deplete intracellular Sam68 and determine if a reduced expression of Sam68 would impact BRCA1 RNA export. To produce MCF7 cell lines with constitutively reduced levels of Sam68 expression, we have transfected MCF cells with pSam68-RNAi or pIL10-RNAi and placed under G418 selection. Sam68 expression in G418 resistant clones was analyzed by Western blot analysis using anti-Sam68 antibodies (Fig. 2). Out of 5 clones analyzed, two of them (#2 and 5) had reduced amounts of Sam68. Expression of endogenous Sam68 in these clones was reduced to 20-40% of the level in MCF7-IL10i cells (#6 and 7) as assessed by immunoblot analysis (Fig. 2). The successful establishment of Sam68 knockdown MCF7 cell lines demonstrates that reduced levels of Sam68 were not toxic.



**Fig. 2: Stable knockdown of Sam68 in MCF7 cells:** Expression levels of Sam68 in stable clones were assessed by Western analysis using anti-Sam68 antibodies. Stable Sam68 knockdown MCF7 cells (clones #1 to 5). MCF-IL10i (clone #6 and 7);  $\beta$ -actin was used as internal control.

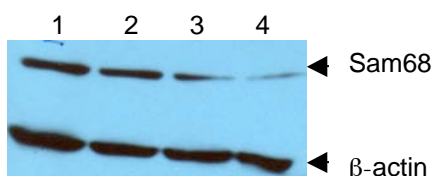
Unfortunately, upon freezing and thawing, the stable Sam68 knockdown cells lost the RNAi vector and cells regained Sam68 expression to normal levels. This was a major setback in our efforts to identify the RNA export of BRCA1. Subsequently, attempts were made to create more clones, but failed. However, we tested the effect of Sam68 knockdown by transient transfection assays using RNAi duplex.

**Knockdown of Sam68 by siRNA duplex in transient assays:** Our objective was to reduce the endogenous Sam68 expression by RNAi duplex in transient transfection assays. As shown in Fig. 1A, we identified one potential target site as described (Elbashir et al., 2001) on human Sam68 mRNA at nt 416 relative to start codon AUG (Fig. 3A). To examine whether the siRNA duplex suppressed the expression of endogenous Sam68, MCF7 cells were transfected with siRNA duplex and analyzed the depletion of Sam68 expression Western blot analysis (Fig. 3B). As shown in Fig. 3B efficient depletion of endogenous Sam68 expression was observed in the cells transfected with Sam68-siRNA (lanes 2 to 4) compared to the cells mutant siRNA (lane1). These results suggest that using RNAi analysis it is possible to knockdown the expression of Sam68 in MCF7 cells.

**A**

Sam68siRNA 416:	5' -GGAUGAUGAGGAGAAUUAC-3' 3' -CCUACUACUCCUCUUAUG-5'
Sam mutant siRNA416:	5' -GGAG <b>C</b> GGGAGGAGAAUUAC-3' 3' -CCU <b>C</b> GGCCUCCUCUUAUG-5'

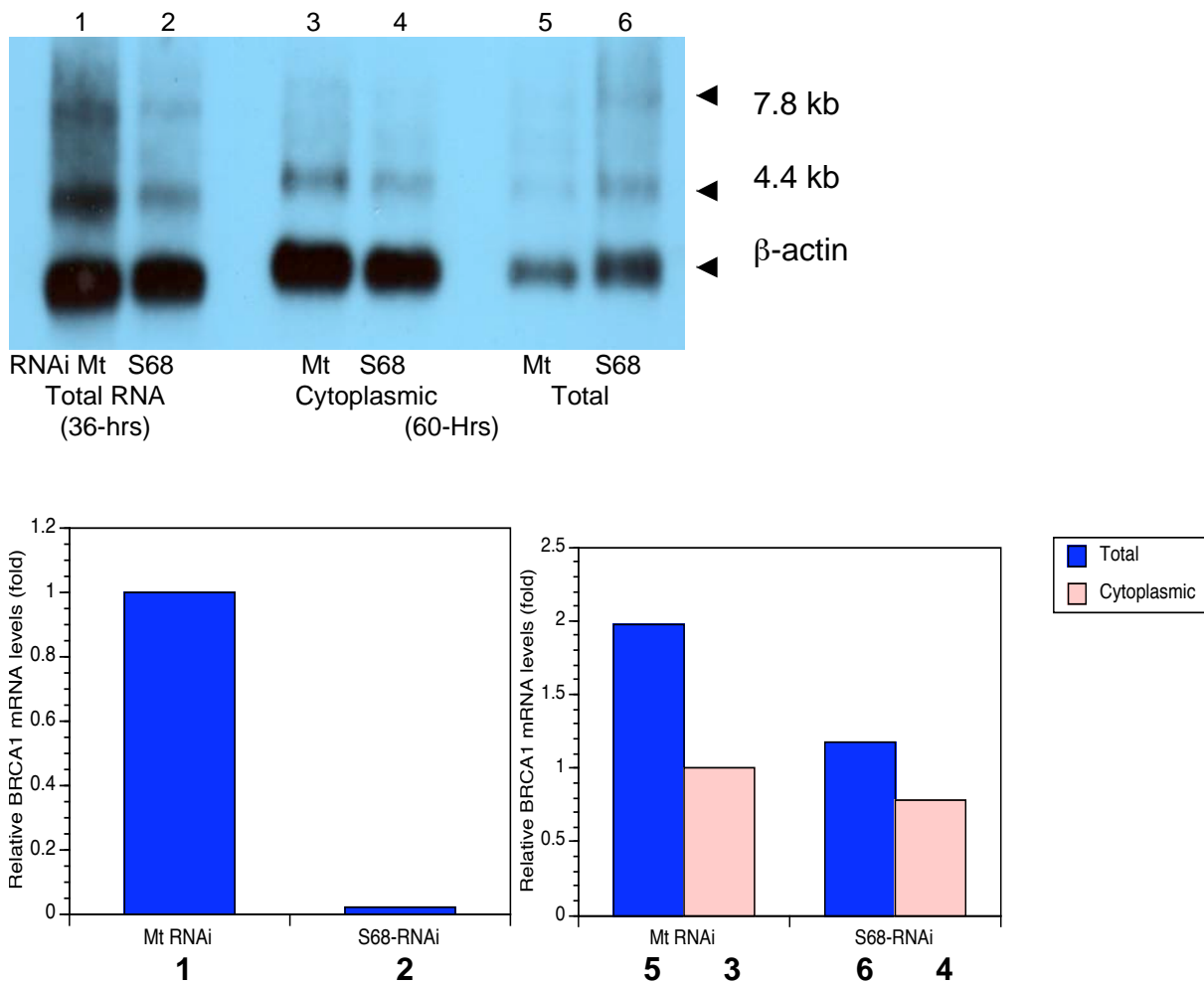
**B**



**Fig. 3. Repression of Sam68 by siRNA analysis.** (A) Sam68-siRNA and mutant siRNA duplexes, (B) Knockdown of Sam68 expression by RNA interference analysis. MCF7 cells were transfected with 100 ng siRNA duplexes, lane 1: mutant RNAi; lanes 2, 3 and 4: Sam68 RNAi, using 6, 3, 6, and 9  $\mu$ l of Hyperfect transfection reagent, respectively. Forty-eight hours post-transfection, cells were lysed and subjected to the Western blot analysis, using Sam68 antibodies. Bold letters in (A) indicate the bases that were mutated.

**Effect of Sam68 knockdown on BRCA1 RNA:** To determine the effect of siRNA-S68 on BRCA1 mRNA levels, MCF7 cells were transfected in the presence wild type or mutant siRNA-S68. At 36-hrs post-transfection, cells were harvested and total RNA was isolated, and subjected to Northern analysis, using BRCA1 <sup>32</sup>P-labeled DNA probe. As shown in Fig. 4A (lanes 1 and 2), BRCA1 mRNA (approximately 7.8 kb) was reduced in siRNA-S68 transfected cells compared to the cells that received mutant-siRNA, which served as control (lane 1), without affecting the  $\beta$ -actin levels. We also observed a band approximately of 4.4 kb size at low levels in Sam68 knockdown cells. It is probably a bi-product of 7.8 kb BRCA1 RNA or a spliced variant of BRCA1 (Lu et al 1996). To further validate the effect siRNA-Sam68 on BRCA1 RNA, we subjected the RNA (that was subjected to the Northern Analysis) to quantitative RT PCR (Fig. 4B). In Sam68 knockdown cells, BRCA1 RNA levels were significantly reduced, which is consistent with the data in the Northern analysis. However, it is unclear whether the reduced level of BRCA1 RNA is because of the transcriptional down regulation or RNA destabilization resulting from the Sam68 knockdown.

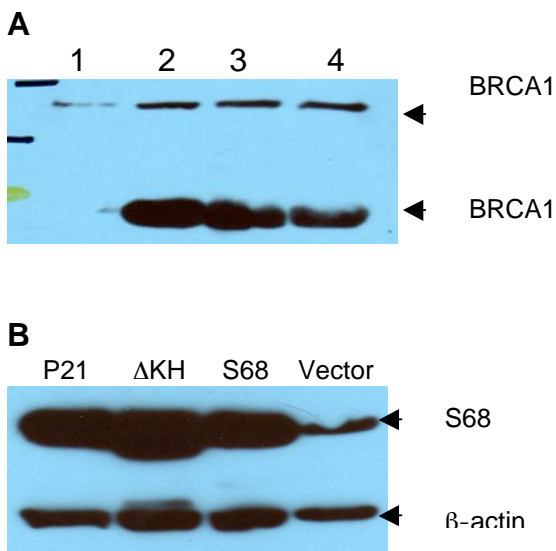
Next to determine whether Sam68 knockdown affects BRCA1 RNA export, we have transfected MCF7 cells with wild type or mutant siRNA-Sam68. At 60-hrs post-transfection, we have harvested the cells and divided into two equal portions. One portion of cells was used to isolate the total RNA and the remainder was used for the cytoplasmic RNA. The RNAs were subjected to the Northern Analysis as described above. The effect of Sam68 knockdown on RNA export was modest, as the difference observed between the cytoplasmic RNA with their respective corresponding total RNA (Fig. 4A; compare lane 3 with 5; and lane 4 with 6) was marginal. Since the Northern analysis data was technically challenging, and inconclusive, alternatively, we sought to analyze the same RNA by quantitative RT PCR. In mutant siRNA transfected cells, the ratio of total versus cytoplasmic RNA was approximately 2:1 (Fig. 4B, lanes 3 and 5). However, in wild type siRNA transfected cells, the ratio between total and cytoplasm was modest (Fig. 4B, lanes 4 and 6). It is possible that at 60-hrs post-transfection, other KH proteins or residual Sam68 may have contributed for the modest effect.



**Fig. 4: Effect of Sam68 knockdown on BRCA1 RNA:** MCF7 cells were transfected with mutant and Sam68-siRNA, independently. At 36 to 60-hours post-transfection, total RNA and/or cytoplasmic RNA was isolated and subjected to Northern blot analysis using the  $^{32}$ P-labeled BRCA1 cDNA as a probe. For internal RNA controls, blots were rehybridized with labeled  $\beta$ -actin cDNA. (A) Northern analysis of total RNA isolated at 36-hrs post-transfection of mutant or S68-RNAi (lanes 1 and 2). At another time point (60-hrs post-transfection) total versus the cytoplasmic RNA was analyzed (lanes 3 to 6). (B) Quantitative real time RT-PCR analysis: We used the BRCA1 forward primer: 5'-TCTGCTCTTCGCGTTGAA-3' and reverse primer: 5'-TCCTTGATCAACTCCAGACA-3'. RNAs of Fig. A were subjected to SYBER quantitative real-time PCR analysis (Badri et al. 2006). Numbers at the bottom of Fig. B corresponds to the lane numbers in Fig. A.

#### Dominant negative mutant of Sam68 inhibits BRCA1 expression:

Mutation in the nuclear localization signal of Sam68 exhibits a dominant negative phenotype on HIV replication (Reddy, 2000). Our objective was to determine the effect of Sam68 dominant negative mutant on BRCA1 expression. To test this possibility, we transfected MCF7 cells with dominant negative mutant (Sam68-P21), or  $\Delta$ KH, or Sam68, or control vectors, independently. At 48-hrs post-transfection, cells were harvested, and cell lysates were prepared and subjected to Western analysis using BRCA1 antibodies (Santa Cruz Biotech, CA). BRCA1 antibody exclusively recognized two bands, one at 120 kDa, and the remainder at 70 kDa (Fig. 5A, upper and lower bands). It is conceivable that these are the degradation products of BRCA1 (approximately 200 kDa). Alternatively, they are the products of BRCA1 spliced variants. Most importantly, in Sam68-P21 transfected cells, these two bands are expressed at reduced levels (Fig. 5A, lane 1) compared to the cells that were transfected with exogenous Sam68,  $\Delta$ KH or control vectors alone (Fig. 5A, lanes 2 to 4).



**Fig. 5: Dominant negative mutant of Sam68 inhibits BRCA1:** MCF7 cells were transfected with various Sam68 mutants and tested the BRCA1 levels by Western blot analysis. (A) lane 1: Sam68-P21, lane 2: Sam68  $\Delta$ KH, lane 3: Sam68 and lane 4: vector alone. Upper arrow correlates with approximately 120 kDa, and the lower band with 70 kDa. To validate the expression levels of mutants Sam68, same blot was probed with anti-Sam68 antibodies and for control we used  $\beta$ -actin.

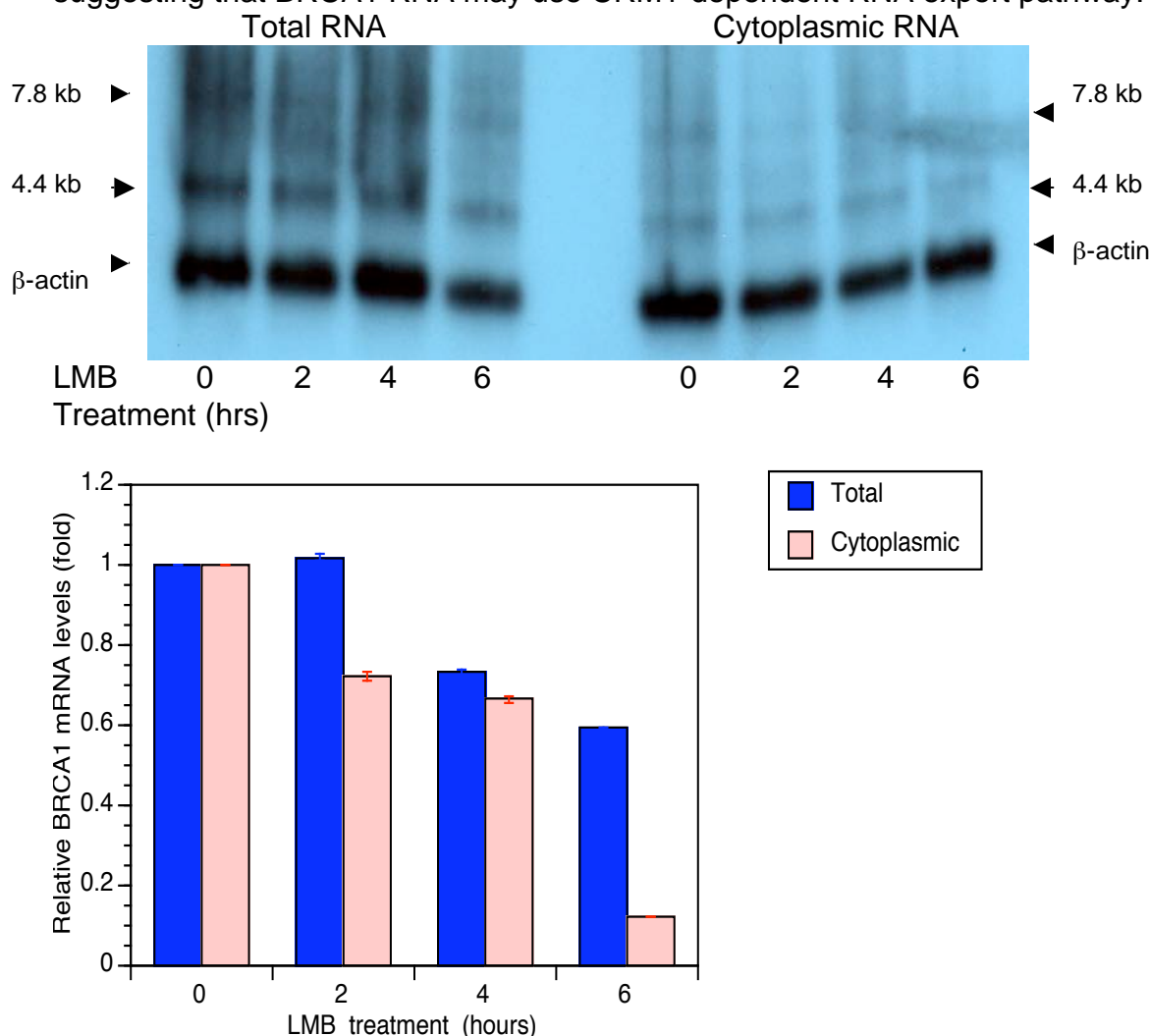
To test whether the observed inhibition is indeed correlated with the expression of Sam68 wild type and its variants, same blot was probed with anti-Sam68 antibodies. The expression levels of wild type Sam68,  $\Delta$ KH and P21 proteins were prolific compared to cells transfected with the control vector. These results clearly suggest that Sam68-P21 inhibits the function of endogenous Sam68, which, in turn, affects the expression of BRCA1. Alternatively, its direct effect on BRCA1 can not be ruled out.

**Aim II. Investigate the RNA export pathway that BRCA1 mRNA uses:** Using LMB, we investigated whether BRCA1 mRNA uses CRM1-dependent or-independent RNA export pathway. Leptomycin B (LMB) was originally identified as an anti-fungal drug from *Streptomyces species*. CRM1 facilitates the export of RNA and proteins containing a nuclear export sequence (NES). Furthermore, CRM1 has been identified as a cellular target of LMB. In the case of RNA export, CRM1 binds to ribonuclear proteins containing the NES motif. LMB drug disrupts that interactions between CRM1 and RNA export cargo, thus inhibiting the RNA export. Since Sam68 participates in the CRM1



dependent pathway in exporting the HIV-1 mRNAs (Li et al. 2002), we tested whether BRCA1 uses CRM1-dependent RNA export pathway. We took advantage of LMB drug to determine whether CRM1 is involved in the BRCA1 mRNA export.

MCF7 cells were maintained in 10% FBS in the presence of LMB (10 ng/ml) and collected cells for the RNA analysis overtime (0, 2, 4 and 6 hrs). Cells were divided into two portions. From one aliquot, we isolated the cytoplasmic RNA and the remainder was used to isolate the total RNA. RNA was subjected to Northern analysis using BRCA1 probe. The 7.8 kb band in total RNA at “0, 2, 4 and 6” hrs was visible when compared to the corresponding bands in cytoplasmic RNA (Fig. 6A). Interestingly, our preliminary results suggest that band at 4.4 kb was not affected with the LMB treatment. If it were to be a spliced variant, it will be interesting that this variant may use another pathway (CRM1-independent). Again, we would like to emphasize on the technically challenging Northern analysis, and therefore, we analyzed the levels of the total and the cytoplasmic BRCA1 RNAs by real time quantitative RT-PCR. As shown in Fig. 6B, at 0-hrs, the cytoplasmic and the nuclear RNAs were comparable. However, at 2 and 4-hrs post treatment, the RNA levels in cytoplasmic fractions was diminished when compared to the nuclear RNA. Most importantly, at 6-hrs post-treatment, the cytoplasmic RNA levels have significantly decreased over the nuclear RNA, suggesting that BRCA1 RNA may use CRM1-dependent RNA export pathway.



**Fig. 6: BRCA1 uses CRM1-dependent RNA export pathway.** MCF7 cells were incubated with leptomycin B (10 ng). Cells were collected at 0 time and thereafter at 2, 4 and 6-hrs post treatment. At 48-hours post-transfection, total or the cytoplasmic RNA was isolated and subjected to (A) Northern blot analysis using the <sup>32</sup>P-labeled BRCA1 cDNA as a probe or (B) RT-PCR analysis. For internal RNA controls, blots were rehybridized with labeled β-actin cDNA. The numerical numbers at the bottom of Fig. B corresponds to the lane numbers in Fig. A.

## KEY RESEARCH ACCOMPLISHMENTS

- Knockdown of Sam68 in MCF7 cells by siRNA
- Sam68 knockdown cells inhibited the BRCA1 mRNA levels, judged by the Northern and quantitative real time RT PCR
- Dominant negative Sam68 mutant inhibits BRCA1 expression
- BRCA1 uses the CRM-1 dependent RNA export pathway

**REPORTABLE OUTCOMES:** Not yet, but probably in the future.

**CONCLUSIONS:** We demonstrated that Sam68, a substrate for Src kinase, is involved in the regulation of BRCA1 gene expression. Sam68 depleted cells express reduced levels of BRCA1 mRNA. Although, our results concretely do not establish a precise step in BRCA1 RNA metabolism, it is our expectation that Sam68 depletion enhances the destabilization of BRCA1 mRNA, thus, inhibiting the RNA export. These results were further validated by the dominant negative mutant strategy, in which BRCA1 is down modulated. In contrast, in Sam68 overexpressing cells, the levels of BRCA1 band(s) were comparable to the control vector transfected cells. Our preliminary results suggest that BRCA1 uses CRM1 dependent RNA export pathway. Since the methodology associated with the project was technically challenging and time consuming, we were unable to define the role of Sam68 in BRCA1 mRNA stability by actinomycin D assays. In the future, it will be of importance to isolate the poly-A mRNA of BRCA1 to precisely determine the role of Sam68 and the RNA export pathway. Once the role of Sam68 in BRCA1 RNA metabolism has been identified, using pharmacological agents that enhance intracellular Sam68 are of beneficiary to maintain the levels of BRCA1, and subsequently prevent the tumor progression. It is also important to determine whether other events, such as post-translational modifications of Sam68, play a role in the regulation of BRCA1 expression. Additionally, it is important to map whether wild type and BRCA1 variants utilize the same RNA export pathway. It is possible that BRCA mutants may use CRM1-independent pathway. It is important to emphasize that the studies with respect to RNA metabolism of the breast cancer associated genes (BRCA1, BRCA2 and aromatase) have been largely overlooked in the breast cancer field. In the future, it is of importance to focus on understanding the role of various RNA export proteins in the RNA metabolism of BRCA1, BRCA2 and aromatase mRNAs, which eventually is expected to shed light on the novel strategies to develop a new class of anti-breast cancer drugs.

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## **APPENDICES: None**